


Anti-CD3 treatment up-regulates programmed cell death protein-1 expression on activated effector T cells and severely impairs their inflammatory capacity

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Summary

T cells play a key role in the pathogenesis of type 1 diabetes, and targeting the CD3 component of the T-cell receptor complex provides one therapeutic approach. Anti-CD3 treatment can reverse overt disease in spontaneously diabetic non-obese diabetic mice, an effect proposed to, at least in part, be caused by a selective depletion of pathogenic cells. We have used a transfer model to further investigate the effects of anti-CD3 treatment on green fluorescent protein (GFP)⁺ islet-specific effector T cells *in vivo*. The GFP expression allowed us to isolate the known effectors at different time-points during treatment to assess cell presence in various organs as well as gene expression and cytokine production. We find, in this model, that anti-CD3 treatment does not preferentially deplete the transferred effector cells, but instead inhibits their metabolic function and their production of interferon- γ . Programmed cell death protein 1 (PD-1) expression was up-regulated on the effector cells from anti-CD3-treated mice, and diabetes induced through anti-PD-L1 antibody could only be reversed with anti-CD3 antibody if the anti-CD3 treatment lasted beyond the point when the anti-PD-L1 antibody was washed out of the system. This suggests that PD-1/PD-L1 interaction plays an important role in the anti-CD3 antibody mediated protection. Our data demonstrate an additional mechanism by which anti-CD3 therapy can reverse diabetogenesis.

Keywords: diabetes; transgenic/knockout mouse; tolerance/suppression/anergy; antibodies.

Introduction

Anti-CD3 treatment was identified over 20 years ago as a promising candidate for treatment of type 1 diabetes, when Chatenoud *et al.* showed that injection of an anti-CD3 antibody could reverse disease in overtly diabetic non-obese diabetic (NOD) mice.¹ Since then, clinical trials have attempted to achieve the same results using non-activating humanized monoclonal anti-CD3 such as teplizumab^{2,3} and oteplizumab.^{4,5} Although some have reported prolonged periods of endogenous insulin production and lower insulin injection dose requirements,^{5–7}

these trials have not succeeded in reversing diabetes long term.⁸ Continued study of all possible tolerogenic mechanisms that can be harnessed by anti-CD3 therapy is essential for establishing how this treatment could be used to its full potential. Treatment with anti-CD3 results in reversal of established diabetes in NOD mice¹ through a combination of early and long-lasting mechanisms. CD3-specific antibodies can induce anergy in T cells through incomplete activation providing signalling through the T-cell receptor (TCR) complex without accompanying co-stimulation,^{9,10} and can also induce T-cell apoptosis.^{9,10} Although anti-CD3 treatment reduces

Abbreviations: BMDC, bone-marrow-derived dendritic cell; GFP, green fluorescent protein; GSEA, gene set enrichment analysis; HbA1c, glycosylated haemoglobin; IFN- γ , interferon- γ ; IL-2, interleukin-2; NOD, non-obese diabetic; PD-1, programmed cell death protein 1; PI3K, phosphoinositide 3-kinase; TCR, T-cell receptor; TGF, transforming growth factor; Th1, T helper 1 subset; Treg, regulatory T

T-cell numbers, the regulatory Foxp3⁺ T-cell (Treg cell) compartment remains undiminished, leading to an increase in the proportion of Treg cells present in blood and secondary lymphoid tissues.^{11,12} A compelling suggestion is that anti-CD3 treatment primarily depletes activated T cells,¹³ which has been supported by studies using ovalbumin-specific cells.^{11,14}

In this study, we have used green fluorescent protein (GFP)-labelled T helper type 1 (Th1) differentiated islet-specific effector cells^{15,16} to study in detail the effects of anti-CD3 treatment on effector T cells with an Fc-mutated (aglycosylated) mouse equivalent of otelixizumab. We observed, in this model, that anti-CD3 treatment did not preferentially deplete these cells, but rather suppressed their capacity to produce interferon- γ (IFN- γ) in response to stimulation and increased their cell surface expression of programmed cell death protein 1 (PD-1). Gene set analysis of microarray data demonstrated fundamental changes in the anti-CD3-treated isolated effector cells compared with control antibody-treated, indicating the down-regulation of genes associated with glycolysis, activation and co-stimulation. This finding offers a further mechanism to explain the therapeutic benefits of anti-CD3 treatment in establishing a tolerogenic milieu.

Materials and methods

Mice and diabetes detection

Female NOD mice, NOD-*scid* mice, BDC2.5 NOD mice¹⁵, NOD-CD2-GFP mice,¹⁷ BDC2.5 \times NOD-CD2-GFP F₁ mice and NOD-Foxp3-GFP mice¹⁸ were bred in the Department of Pathology, University of Cambridge and maintained under specific pathogen-free conditions. The mice are housed in individually ventilated cages with free access to standard chow and water. Diabetes was detected using Diastix reagent strips (Bayer Diagnostics, Basingstoke, UK) and confirmed by a blood glucose measurement of > 13.3 mM, using a Breeze2 blood glucose meter (Bayer). This study was carried out in strict accordance with UK Home Office project licence regulations (Project Licence number 80/2442 and 70/8442) after approval by the Ethical Review Committee of the University of Cambridge.

Antibody treatment

Aglycosyl anti-CD3 antibody. The non-Fc receptor-binding anti-mouse CD3 antibody (agly-anti-CD3) was generated through genetic engineering in the Waldmann Laboratory, University of Oxford. It consists of the antigen-binding variable domain of the anti-mouse CD3 KT3 clone fused to mutated (non-FcR binding) human IgG1 heavy and κ light chains. Chimeric KT3-1.1 aglycosyl IgG1 antibody mRNA was prepared from the cells of the

hybridoma KT3-1.1 and cDNA was prepared by anchor-tailed PCR. Splicing by overlap extension PCR was then carried out for the light chain using primers (1) MR1KT3 5'-

TGTGACCCTGCTGCTCTGGGTGTCTGGTGCCTGTG-CAGACATCCAGATGACCCAGTCTCC and (2) KT3VL-CK 3'-TGCAGCCACAGTCCGTCTCAATTCCACCTTGG TGCCTCC for VL and primers (3) KT3VL-CK 5'-GAAT TGAGACGGACTGTGGCTGCACCATCTGTCTTCATC and (4) huCKH111 3'-TGAGCCAAGCTTTCAACACTCT CCCCTGTTGAAGCTCTT for Ck. Primers 1 and 4 were then used to assemble light-chain constructs. Splicing by overlap extension PCR was also carried out for the heavy chain using primers (5) KT3VhH111 5'-TCTAG-TAAGCTTGCCGCCACCATGGACTTCAGGCTCAGCCT GGCTTTC and (6) KT3CH1 3'-TTGCTGGAGGCTGAG-GAGACTGCGACCATGACTCCTTGG for VH and primers (7) KT3CH1 5'-CGCAGTCTCCTCAGCCTCCACCA AGGGCCCATCGGTCTT and (8) huCH3ER1 3'-TAGATCGAATTCGGGGCCGTCGCACTCATTACCCG-GAGACAGGG for constant region. Primers 5 and 8 were then used to assemble a heavy-chain aglycosyl IgG1 construct. The amplified light-chain fragment was cloned into the *Hind*III site of PEE12 vector (Lonza, Basel, Switzerland) and the heavy chain was cloned into the *Hind*III and *Eco*RI sites of PEE12. The resulting plasmids were co-transfected by electroporation into the cell line NSO and stable transfectants were selected in glutamine-free Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA). Positive clones were selected by ELISA for binding to anti-human IgG1 and by FACS for binding to EL4 cells. Antibody was purified by Protein A (GE Healthcare, Chalfont St Giles, UK) and tested for endotoxin. The antibody that recognizes human CD3 (otelixizumab) was used as a non-binding isotype control.

Anti-PD-L1 antibody. Anti-mouse programmed cell death ligand 1 (PD-L1) antibody (clone MIH5, grown in-house) was administered intraperitoneally once at a dose of 2 mg.

Th1 differentiation for *in vitro* studies and adoptive transfer

CD4⁺ CD62L^{hi} CD25⁺ B220⁺ T cells were isolated by cell sorter (MoFlo; Beckman Coulter, Brea, CA) from 5-week-old BDC2.5 TCR transgenic NOD mice and differentiated into Th1 cells by culturing them in Iscove's modified Dulbecco's medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, 50 μ M β -mercaptoethanol, L-glutamine (2 mM) and 50 U/ml penicillin and streptomycin (Gibco) with plate-bound anti-CD3 (2 μ g/ml, clone 145-2C11, grown in-house), soluble anti-CD28 (10 μ g/ml; BD Pharmingen, Franklin Lakes, NJ) and interleukin-2 (IL-2; 100 U/ml), IL-12 (10 ng/ml) and

interferon- γ (IFN- γ ; 100 U/ml) (all from Peprotech, Rocky Hill, NJ) for 4 days at 37° with 5% CO₂. Afterwards, the production of IFN- γ was checked by specific ELISA (R&D Systems, Minneapolis, MN) and/or intracellular staining.

Proliferation studies

For determination of *in vivo* proliferation, BDC2.5 CD4⁺ T cells were stained with CFSE (5 μ M) and injected intravenously into 6-week-old NOD mice that had received a Th1 cell transfer 1 week previously and subsequent treatment with agly-anti-CD3 or control antibody for 4 days. Seventy-two hours after injection of the CFSE-labelled cells the indicated lymph nodes were harvested and the cells were stained for surface markers. For determination of *in vitro* proliferation, BDC2.5 CD4⁺ T cells were stained with CFSE (5 μ M) and co-cultured for 72 hr with whole pancreatic lymph nodes from 6-week-old NOD mice, which had received a Th1 cell transfer 1 week previously and had subsequently received treatment with agly-anti-CD3 or control antibody for 4 days.

Co-culture with bone-marrow-derived dendritic cells

Bone marrow was harvested from the femurs of NOD mice and cultured in 50 ml Dulbecco's modified Eagle's medium supplemented as above plus 10 ng/ml granulocyte-macrophage colony-stimulating factor (Peprotech) for 10 days. For co-culture the cells were pulsed with BDC2.5 mimotope RTRPLWVRME (1 μ g/ml; Cambridge Peptides, Birmingham, UK) or not. For co-culture, the BDC2.5 Th1 differentiated cells were pre-incubated with the indicated concentration of agly-anti-CD3 or control antibody for 1 hr at 4° and then washed. Bone-marrow-derived dendritic cells (BMDC; 2.5 \times 10⁵ cells per well) and Th1 cells (5 \times 10⁵ cells per well) were then co-cultured in 24-well plates for 48 hr, after which supernatants were collected for cytokine analysis and cells were collected for analysis of CD80 expression.

Supernatant cytokine analysis

Cytokine levels in cell culture supernatants were detected using a cytometric bead array (eBioscience, San Diego, CA) according to the manufacturer's instructions. The R&D Systems ELISA kit for detection of IL-12 (p40) was used according to the manufacturer's instructions.

Antibodies and flow cytometry

Cell suspensions from lymph nodes and spleen were prepared by dispersion between glass slides. Intestine and pancreas were manually dispersed, and then further digested with collagenase solution (0.5 mg/ml; Sigma, St

Louis, MO) as previously described.¹⁹ All cell preparations were resuspended in FACS buffer (PBS with 0.5% BSA) and incubated with Fc-block (eBioscience). Cell death was assessed with 7AAD (BD Bioscience, Franklin Lakes, NJ). Foxp3 was detected using intracellular cytokine staining kit and anti-Foxp3 from eBioscience. Data were collected on a Cyan Cell Cytometer (DAKO, Santa Clara, CA) and analysed using FLOWJO (Tree Star Inc., Ashland, OR). For intracellular cytokine staining, the cells were stimulated with PMA (50 ng/ml) and ionomycin (2000 ng/ml) for 5 hr. Brefeldin A (5 μ g/ml) was added for the last 3 hr and stained with allophycocyanin-conjugated anti-IFN- γ (XMGI.2) or allophycocyanin-conjugated IgG1 isotype controls (BD Pharmingen). Mitotracker and mitosox assays (both from Thermo Fisher, Waltham, MA) were performed according to the manufacturer's instructions, with incubation for 30 min at 37° (mitotracker) or 10 min at 37° (mitosox).

Immunofluorescence

Pancreata were fixed in 4% paraformaldehyde for 72 hr, dehydrated in sucrose and mounted in OCT. Ten-micron sections were cut on a Leica cryostat onto Polysine slides (VWR), air-dried and fixed again for 10 min in acetone. Guinea-pig-anti-insulin antibody (DAKO) was detected with anti-guinea-pig Alexa 546 (Molecular Probes, Eugene, OR). Anti-CD4 (BD) was detected with anti-rat Alexa 488 (Molecular Probes). GLUT-1 in cells was detected using ab15309 (Abcam, Cambridge, UK) and Alexa 647 anti-rabbit (Molecular Probes) after permeabilization with PBS-Triton-X. Nuclei were visualized with DAPI (Molecular Probes). The sections were viewed with a confocal microscope (Zeiss, Oberkochen, Germany) and processed using ZEN software.

Microarray

RNA was assessed for concentration and quality using a SpectroStar (BMG Labtech, Aylesbury, UK) and a Bioanalyser (Agilent Technologies, Cheshire, UK). Microarray experiments were performed at Cambridge Genomic Services, University of Cambridge, using the MouseWG-6 v2 Expression BeadChip (Illumina, Chesterford, UK) according to the manufacturer's instructions. Briefly, total RNA was amplified using the Ovation Pico WTA v2 kit (NuGEN Technologies, Leek, Netherlands) and subsequently labelled using the BiotinIL kit (NuGEN Technologies) following the manufacturer's instructions. The concentration, purity and integrity of the resulting cRNA were measured using the Nanodrop ND-1000 (Thermo Scientific, Paisley, UK) and by BIOANALYSER. Finally, cRNA was hybridized to the MouseWG-6 v2 BeadChip and scanned using the Bead Array Reader (Illumina).

Raw data were loaded into R using the LUMI package from bioconductor²⁰ (<http://bioconductor.org>) and divided into subsets according to the groups being compared; only the samples involved in a given comparison are used. Subsets were then filtered to remove any non-expressed probes using the detection *P*-value from Illumina. Across all samples, probes for which the intensity values were not statistically significantly different ($P > 0.01$) from the negative controls were removed from the analysis. The data were transformed using the Variance Stabilization Transformation (VST²¹) from LUMI and then normalized to remove technical variation between arrays using quantile normalization. Comparisons were performed using the LIMMA package²² with results corrected for multiple testing using a False Discovery Rate (FDR) correction. Finally, the quality of the data was assessed along with the correlations between samples within groups.

Gene set enrichment analysis (GSEA) was performed as previously described.²³ Molecular signatures (<http://www.broad.mit.edu/gsea>) interrogated included Hallmark (H), curated (C2) and gene ontology (C5) groups including KEGG, Reactome and Biocarta. Molecular signatures were considered to be significantly associated with treated or untreated groups if they had a nominal *P* value of <0.001 and a false discovery score of <0.25 . We acknowledge our use of the gene set enrichment analysis, GSEA software, and Molecular Signature Database (MSigDB).²³ The raw data are available at <https://www.repository.cam.ac.uk/handle/1810/254543>

Statistical analysis

Differences between groups were tested using Student's *t*-test or non-parametric Mann–Whitney *U*-test as indicated. Differences between animals regarding diabetes incidence were tested using the Log rank survival test, with *P*-values displayed within the relevant figure or legend. Analysis was performed using GRAPHPAD PRISM software (GraphPad Prism, San Diego, CA).

Results

Agly-anti-CD3 treatment prevents Th1-differentiated pathogenic islet-specific CD4⁺ T cells from causing disease but does not preferentially deplete them

Aglycosylated anti-CD3 antibody can activate mouse T cells and cause cytokine production when used plate bound in *in vitro* assays (see Supplementary material, Fig. S1a), but soluble antibody fails to activate T cells in co-culture with BMDC, confirming that it does not bind Fc-receptors (see Supplementary material, Fig. S1b). As the antibody cannot bind murine Fc-receptors it does not cause release of large amounts of pro-inflammatory

cytokine, so avoiding an antibody-induced cytokine storm (see Supplementary material, Fig. S1c). Intravenous injection of this antibody reversed diabetes in NOD mice when injected at sufficiently high concentrations, whereas a low concentration of 0.5 µg/dose given for five daily doses did not have an effect on disease (see Supplementary material, Fig. S2). As shown previously with non-FcR binding anti-CD3 antibodies, treatment with agly-anti-CD3, but not isotype control antibody, decreased the numbers of T cells but spared the Foxp3⁺ Treg population (see Supplementary material, Fig. S3).

To investigate whether the observed preferential depletion of non-Treg cells was caused by a selective depletion of activated, pathogenic cells we used an adoptive transfer system, where GFP⁺ labelled islet-specific Th1 differentiated BDC2.5 CD4⁺ effector cells were transferred into 5-week-old female NOD mice (Fig. 1a). These mice were at least 5 weeks away from developing diabetes spontaneously, ensuring that the transferred Th1 cells could be held responsible for rapid progression to diabetes. Treatment with agly-anti-CD3 for 3 days prevented progression to diabetes, and prevented immune cell infiltration into the islets (Fig. 1b,c). As the transferred effectors were GFP⁺, we could seek these cells in spleen, pancreatic lymph nodes and pancreas to assess if they had been preferentially depleted by the treatment. Although the absolute numbers of non-Treg cells had decreased in line with previous results, the GFP⁺ CD4⁺/GFP[−] CD4⁺ ratio (Fig. 2a) was the same in both agly-anti-CD3 treated mice and the isotype controls (Fig. 2b). This was the case at any time-point we investigated after adoptive transfer (Fig. 2c). We ascertained that the transferred effector cells had not converted into FoxP3⁺ Treg cells, through use of Th1 differentiated BDC2.5 cells from a donor mouse expressing GFP under the Foxp3 promoter (see Supplementary material, Fig. S4). In fact, it became obvious that these cells, with potential to initiate disease, disappeared quickly after adoptive transfer, and were all-but-gone 6 days after injection regardless of whether the recipient mice received agly-anti-CD3 or isotype control treatment (Fig. 2c).

Agly-anti-CD3 treatment down-regulates the expression of genes involved in glycolysis and co-stimulation and suppresses production of IFN-γ in pathogenic islet-specific T cells

To gain deeper understanding of how treatment with agly-anti-CD3 affects the transferred Th1 cells to prevent them from precipitating diabetes, we investigated changes in their gene expression. We performed the same transfer as described in Fig. 1(a), treating the recipient mice with either agly-anti-CD3 or isotype control antibody, and sorted the GFP⁺ cells from the pancreatic draining lymph nodes 4 days following adoptive transfer. We then

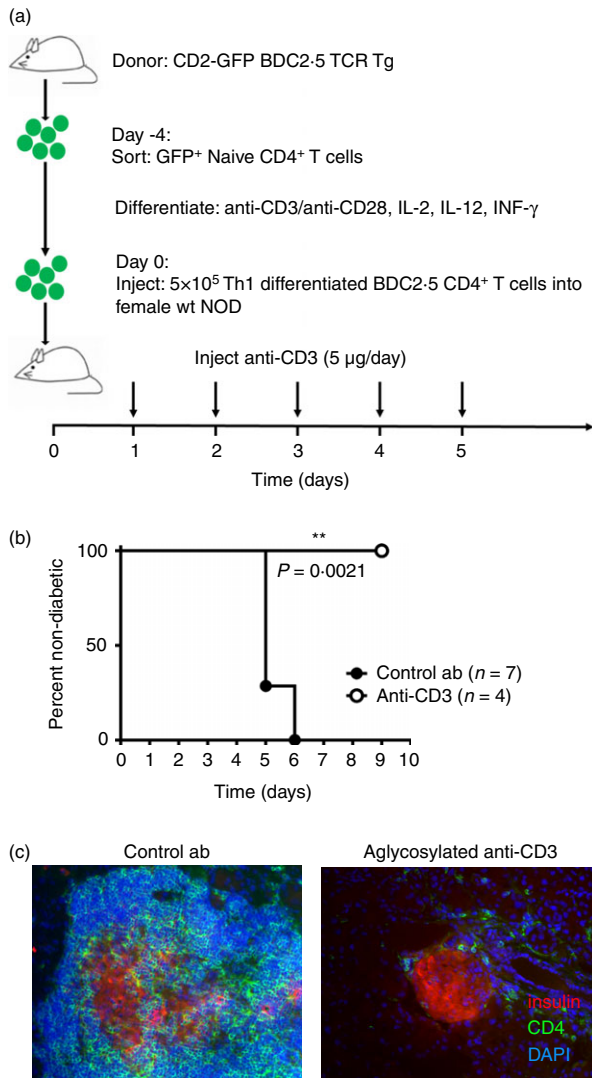


Figure 1. Agly anti-CD3 protects non-obese diabetic (NOD) mice from developing diabetes after adoptive transfer of T helper type 1 (Th1) -differentiated BDC2.5 T cells. Adoptive transfer of GFP⁺ labelled Th1-differentiated BDC2.5 CD4⁺ T cells into 5-week-old NOD female mice, followed by three daily injections of agly-anti-CD3 (5 μ g/day) (a) led to diabetes by day 5 in the isotype control treated mice while agly-anti-CD3 treated mice remained healthy (b). Histological examination of the pancreas is shown after treatment with either agly-anti-CD3 (right) or control antibody (left), with insulin stain in red (Alexa 546), CD4 in green (Alexa 488) and DAPI in blue (c). The difference in diabetes incidence was tested using the log-Rank survival test, (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

isolated RNA and performed microarray analyses to detect any differences in gene expression between Th1 effector cells isolated from agly-anti-CD3-treated mice, and those isolated from control mice (see Supplementary material, Fig. S5). GSEA²³ of microarray data of GFP⁺ transferred Th1 cells revealed several significantly different molecular signatures in T cells from protected mice compared with isotype-treated controls. Fourteen molecular

signatures were associated with untreated mice compared with nine from anti-CD3-treated mice (see Supplementary material, Table S1). Of note, in comparison with anti-CD3-treated cells, T cells from control 'isotype'-treated mice had the transcriptional hallmark of activated cells. These cells were enriched for glycolytic genes (Fig. 3a) and components of the IL-6 and CD40 pathways (Fig. 3b–d) including CD40 ligand, signal transducer and activator of transcription 5, phosphoinositide 3 kinase (PI3K) and mitogen-activated protein kinase.

In order to examine the effects of anti-CD3 treatment on cell behaviour, we performed the same transfer and sort as described previously (Fig. 1a), and re-stimulated the cells for 4 hr with PMA and ionomycin. This analysis revealed complete suppression of IFN- γ production (Fig. 4a) in the anti-CD3-treated group compared with the isotype control. Studies in central nervous system inflammation have suggested that anti-CD3 treatment may induce IL-10 production in previously pathogenic cells, and that this may be a mechanism for the protective effects seen.²⁴ However, in our experimental system we could find no evidence that the potentially pathogenic transferred GFP⁺ effector T cells had acquired IL-10 secretion (see Supplementary material, Fig. S6). GLUT-1 is mostly maintained within the cell cytoplasm, to be transported to the surface in response to activation. Analysis of surface GLUT-1 revealed that GFP⁺ cells isolated from anti-CD3-treated mice expressed lower levels of cell surface GLUT-1 (Fig. 4b), and lower levels of intracellular GLUT-1 (Fig. 4c), than their isotype-treated littermates. In addition, GFP⁺ cells from the anti-CD3-treated group were smaller (Fig. 4d). Both these observations indicate a less activated status. No difference in their mitochondrial content or their production of superoxide (Fig. 4e) was discernible.

Agly-anti-CD3 inhibits T-cell-mediated activation of antigen-presenting cells

The adoptively transferred cells disappeared quickly, and 6 days after transfer they were mostly undetectable (Fig. 2c) even in the islet infiltrates, indicating that the transferred cells had simply detonated the anti-islet pathology and then disappeared. At 5 weeks of age there is normally only limited lymphocyte infiltration in the NOD mouse islets, so it is clear that the transferred cells somehow accelerated disease presumably by recruitment and activation of host islet-reactive cells. One way this could have happened is by the licensing of host dendritic cells,^{25–27} a notion further supported by the changes in the CD40–CD40 ligand pathway observed in the GSEA. We tested this by investigating how pre-incubation with agly-anti-CD3 affected the activation of mimotope-presenting BMDC by Th1 differentiated BDC2.5 T cells *in vitro*. We found that BMDC cultured with Th1 BDC2.5 pre-treated with agly-anti-CD3 failed to up-regulate the co-stimulatory

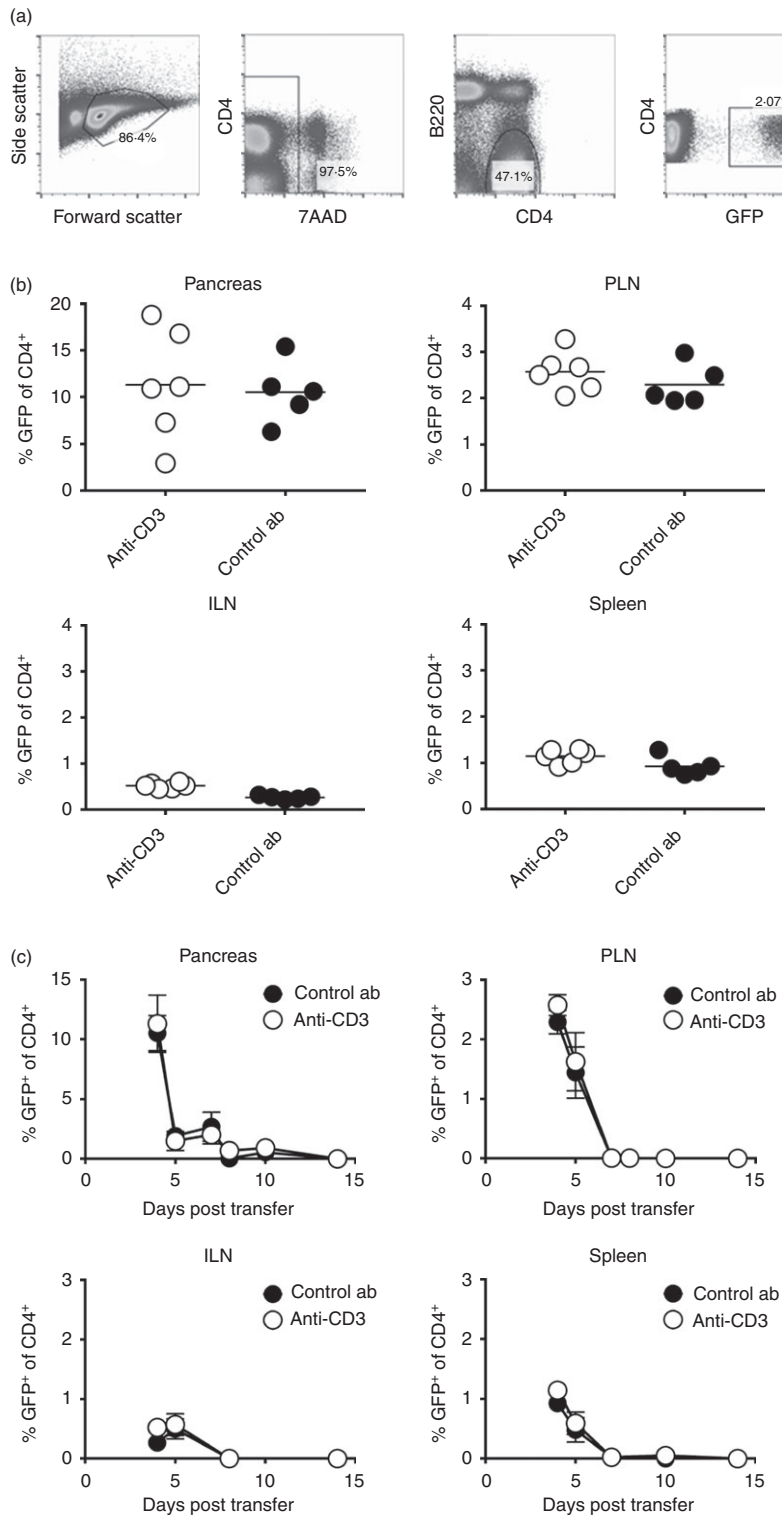


Figure 2. Agly anti-CD3 does not cause preferential depletion of transferred pathogenic cells. The gating strategy for the analysis of the percentage of known pathogenic GFP⁺ CD4⁺ 7AAD⁻ B220⁻ is shown in (a), and the percentage of GFP⁺ T cells within the CD4⁺ T-cell compartment in the indicated organs at 4 days after adoptive transfer (b). The percentage of GFP⁺ T cells within the CD4⁺ T-cell compartment in inguinal lymph nodes, spleen, pancreatic lymph nodes and pancreas at various time-points after adoptive transfer is indicated in (c). Each data point in (b) represents one mouse, and the experiment was performed at least twice for each time-point. The data points in (c) represent an average of at least five mice. Differences between groups were tested using Student's *t*-test.

molecule CD80 to the same extent as either positive controls cultured without any antibody, or controls where the BDC2.5 T cells had been pre-treated with the isotype antibody, which recognizes human CD3 (Fig. 5a). In addition, the BMDC exposed to pre-treated Th1 differentiated BDC2.5 T cells produced less IL-12 (Fig. 5b).

To investigate if treatment with agly-anti-CD3 affected islet antigen presentation *in vivo*, we performed the same transfer as described in Fig. 1(a), and then either tested the capacity of pancreatic lymph node-derived antigen-presenting cells to present endogenous antigen to naive BDC2.5 CD4 T cells *ex vivo* (Fig. 5c) or completely

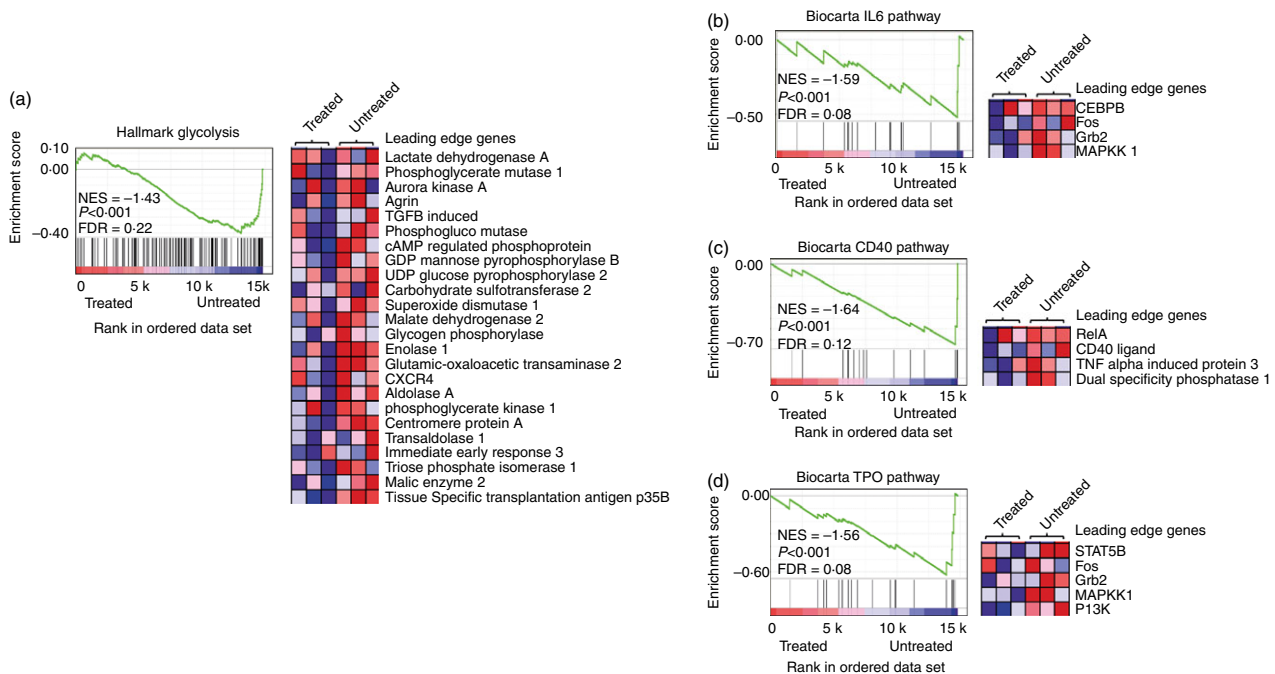


Figure 3. Gene set enrichment analysis of microarray data from donor T helper type 1 (Th1) cells after treatment with either isotype antibody or agly-anti-CD3. wild-type non-obese diabetic (Wt NOD) female mice received 5×10^5 Th1 differentiated BDC2.5 CD4⁺ T cells, and were then treated with isotype control antibody or agly-anti-CD3 as described in Fig. 1. Four days after transfer, the GFP⁺ cells were sorted from the pancreatic lymph nodes of the mice, and the sorted cells were prepared for microarray analysis. Each agly-anti-CD3 sample was pooled from three or four mice, and each control sample was pooled from two or three mice. Molecular signature data sets analysed included hallmark data sets (a) and curated gene sets (b-d). Glycolysis genes are enriched in untreated T cells (a), the leading edge set of genes, those contributing most to the enrichment score, is shown in the heat map. The enrichment score plot indicates the degree of correlation of members of the data set with genes in the molecular signature. In this case a negative correlation is shown, indicating enrichment of glycolysis genes in the isotype-treated groups. Components of the IL-6 (b), CD40 (c) and Thrombopoietin (TPO) (d) pathways are also associated with isotype-treated T cells.

in vivo through injection of the naive CFSE-labelled BDC2.5 cells 3 days after the last antibody injection (Fig. 5d). We found that BDC2.5 CD4⁺ T-cell proliferation was modestly but significantly decreased in agly-anti-CD3-treated pancreatic lymph nodes both when assessed *ex vivo* (Fig. 5c) and *in vivo* (Fig. 5d).

Agly-anti-CD3 increases expression of PD-1 on pathogenic islet-specific T cells

Expression of PD-L1 on antigen-presenting cells is an important regulator of anti-islet immune responses,^{13,28–31} and blockade of this interaction can precipitate diabetes in prone strains.^{29,32} Furthermore, blockade of PD-1/PD-L1 increases the interaction time between T cells and antigen-presenting cells, so indirectly enhancing activation.³³ We used the same experimental set up described in Fig. 1(a), and found that anti-CD3 treatment leads to around an eight-fold increase in the expression of PD-1 on the GFP⁺ donor-derived formed Th1 cells (Fig. 6a). Anti-PD-L1-accelerated diabetes in wild-type NOD mice could be delayed by anti-CD3 treatment, but this protective effect was lost if anti-CD3 therapy was discontinued while PD-

L1 antibody remained in the system (Fig. 6b), the half-life of anti-PD-L1 antibody being c. 7 days.³⁴ Only anti-CD3 treatment that continued beyond the point when anti-PD-L1 was sufficiently purged from the system was sufficient to establish lasting tolerance and euglycaemia (Fig. 6c).

Discussion

Anti-CD3 treatment has shown efficacy in certain clinical trials, with prolongation of time to dependence on exogenous insulin administration in treated patients, and lower levels of HbA1c. The fact that anti-CD3 treatment in humans failed to mimic the complete remission seen in diabetic mice initially disappointed the type 1 diabetes community. However, detailed analysis of the treated cohorts indicates that success might be increased through focus on particular subgroups such as younger patients⁷ or in patients identifiable by lower HbA1c and insulin requirement at the beginning of the study.^{6,35} It is also clear that too low an antibody dose fails to achieve any positive effects,³⁶ which, as we demonstrate in this paper, is also true in NOD mice. There is a growing consensus to consider combination therapies targeting different aspects

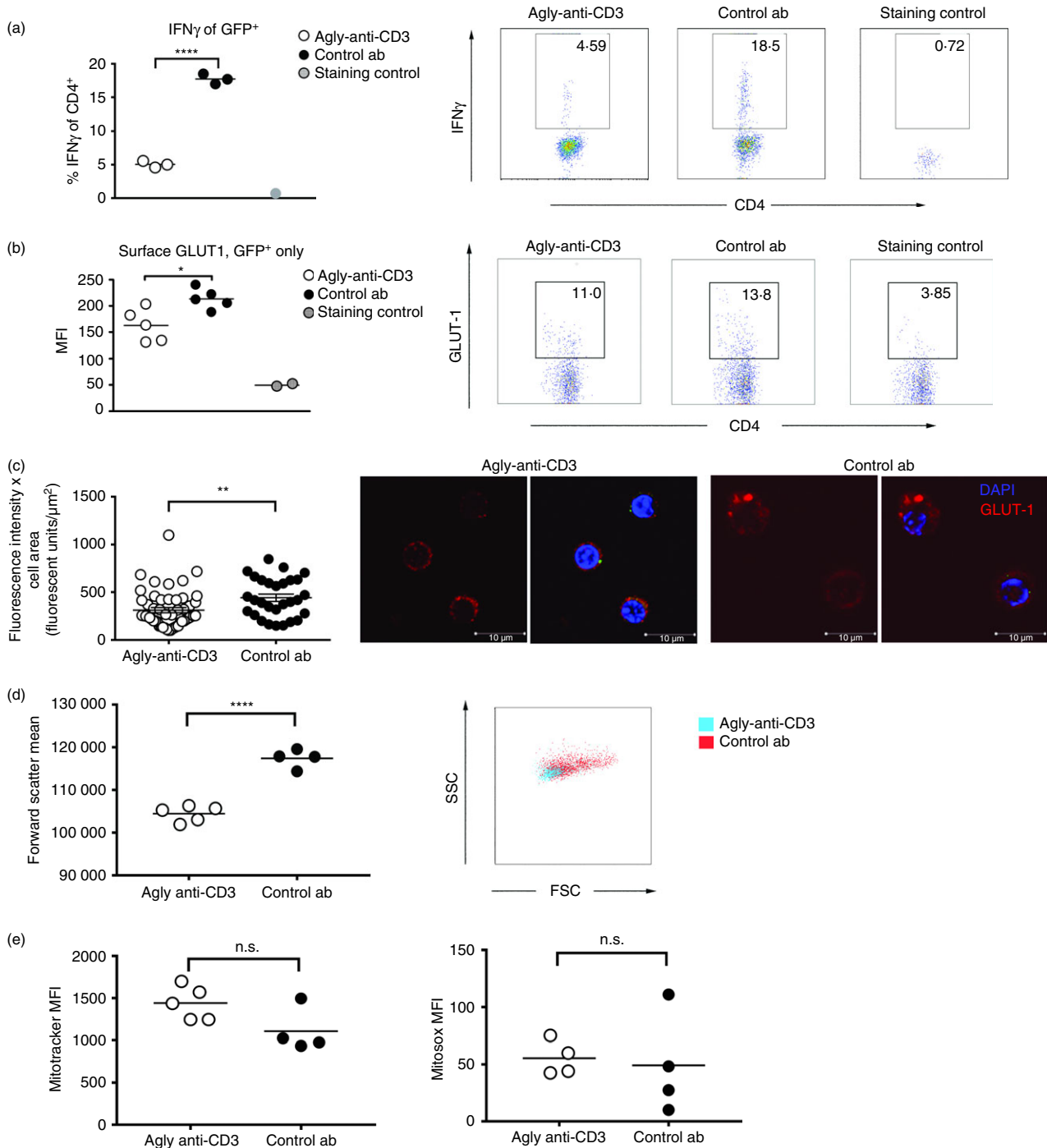


Figure 4. Functional studies of T helper type 1 (Th1) cells after treatment with either isotype control antibody or agly-anti-CD3. Wild-type non-obese diabetic (wt NOD) female mice received 5×10^5 Th1 differentiated BDC2.5 CD4 $^{+}$ T cells, and were then treated with isotype control antibody or agly-anti-CD3 as described in Fig. 1. Four days after transfer the GFP $^{+}$ cells were sorted from the pancreatic lymph nodes of the mice. The cells were assessed for interferon- γ (IFN- γ) (a), surface GLUT-1 (b), intracellular GLUT-1 content (c), size through forward scatter (d), mitochondrial content with mitotracker and mitochondrial superoxide production with mitosox (e). Each data point represents one mouse except in (a), where each data point is a pool of two or three mice. Each experiment was repeated at least twice. Differences between groups were tested using Student's *t*-test.

of disease, such as inflammation and beta cell function, in order to improve outcome,³⁷ and that anti-CD3 treatment could be a useful component in such an approach.^{37–39}

Previous work has described a preferential depletion of Th1 differentiated cells after anti-CD3 treatment.^{11,14} In this study we wanted to determine in detail which

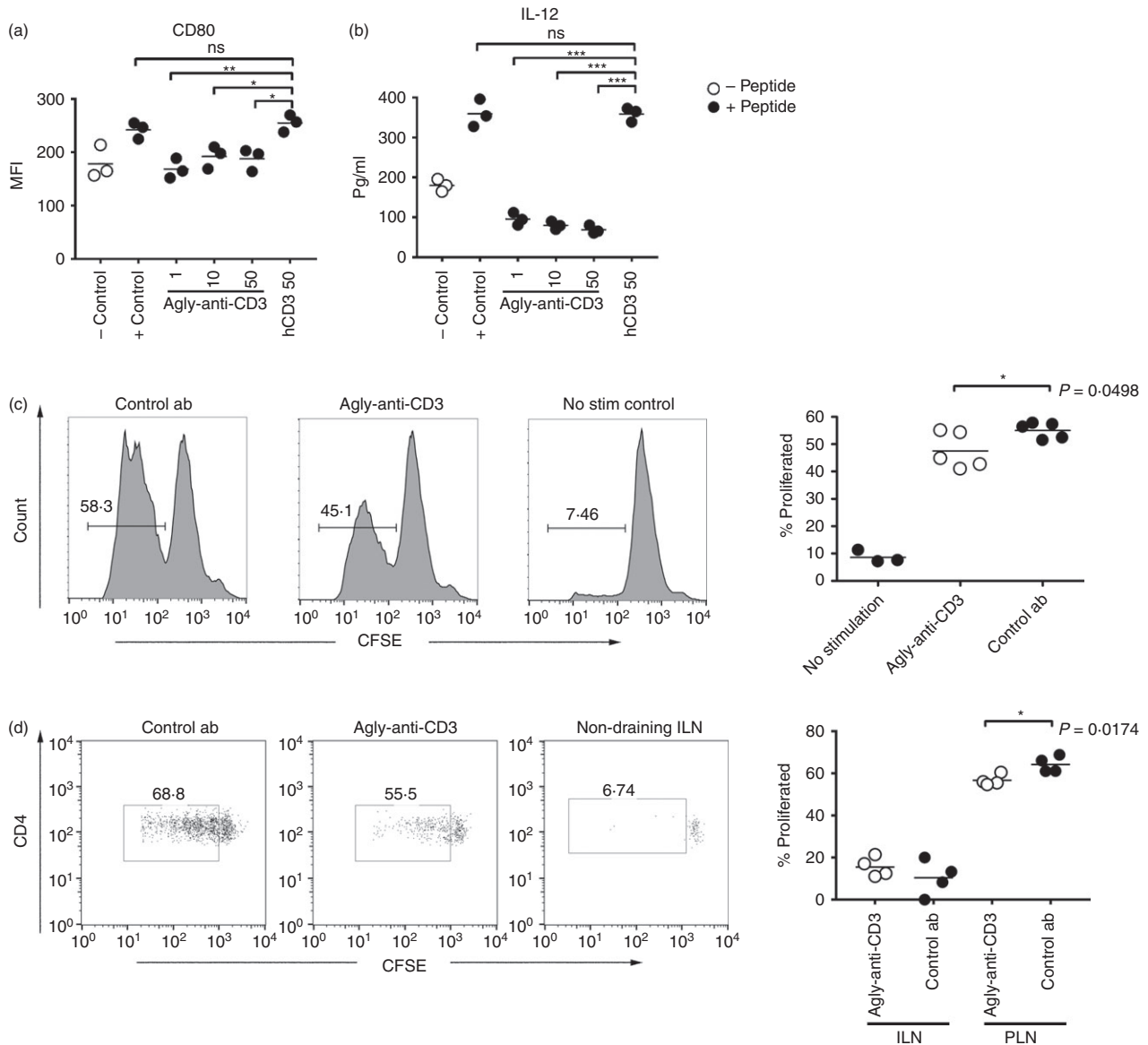


Figure 5. Agly-anti-CD3 inhibits T cells from activating antigen-presenting cells (APC). Pre-incubation with agly-anti-CD3 prevents T helper type 1 (Th1) differentiated BDC2.5 T cell from activating bone marrow-derived dendritic cells (BMDC). Levels of co-stimulatory molecule CD80 on the co-cultured BMDC (a) were measured by flow cytometry, and interleukin-12 (IL-12) in the culture medium was detected by ELISA (b). Controls are BMDC cultured alone, positive control cultures have Th1 cells added without pre-incubation with antibody, and in hCD3 cultures the Th1 cells have been pre-incubated with the isotype control antibody which binds human, but not mouse CD3. Mice were injected and treated as in Fig. 1, and pancreatic nodes used as antigen-presenting cells to CFSE-labelled BDC2.5 CD4⁺ cells *in vitro* (c) or *in vivo* (d) to assess proliferation. Differences between groups were determined using the non-parametric Mann-Whitney test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). The results are representative of at least three experiments.

'activated' cells disappear through use of GFP-labelled Th1-differentiated islet-specific BDC2.5 CD4⁺ T cells, injected into young, pre-diabetic female NOD mice. In this case, we know that the peptide recognized by the BDC2.5 cells is presented in the pancreatic lymph nodes,¹⁶ and that these cells cause rapid progression to diabetes after transfer.⁴⁰ Interferon- γ is necessary for CD4⁺ T-cell-mediated beta cell destruction, as demonstrated by studies blocking the cytokine⁴¹ as well as

studies in mice without a functional receptor.⁴² We found that the transferred cells disappeared quickly after transfer, and were mostly gone around 6 days after injection when disease became apparent. The massive infiltrates around the pancreatic islets consisted almost entirely of endogenous T cells and recruited mononuclear cells. It appears therefore, that the transferred activated cells precipitated disease, but did so within a very short time window of activation. The composition of the CD4⁺ T-cell

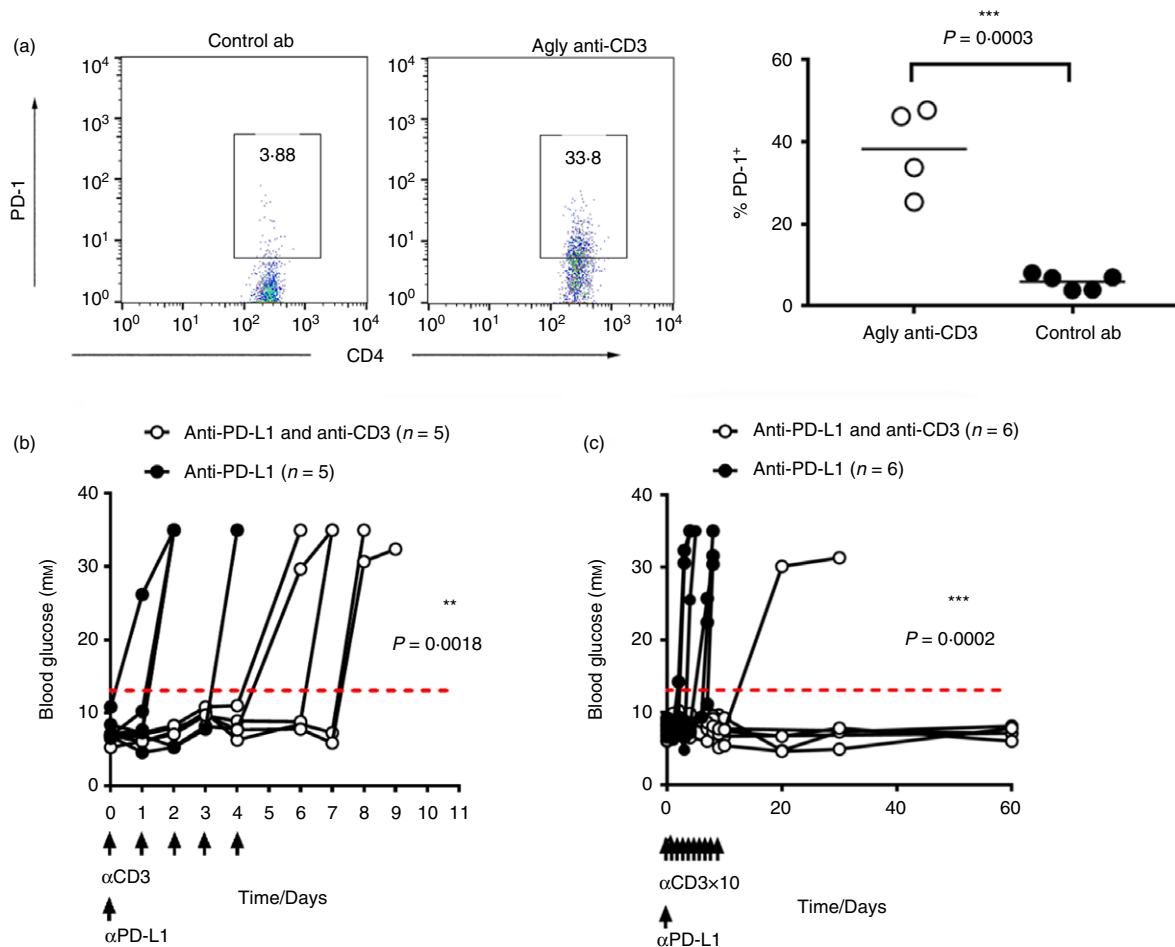


Figure 6. Agly anti-CD3 treatment increases programmed death 1 (PD-1) expression on islet-specific effector T cells, and blockade of PD-1 activation overcomes agly anti-CD3-induced protection from diabetes. Wild-type non-obese diabetic (Wt NOD) female mice received 5×10^5 T helper type 1 (Th1) differentiated BDC2.5 CD4⁺ GFP⁺ T cells, and were then treated with isotype control antibody or agly-anti-CD3 as described in Fig. 1. Four days after transfer the cells in the pancreatic lymph nodes were assessed for PD-1 expression (a). Injection of 2 mg of anti-PD-L1 antibody in pre-diabetic NOD mice combined with short term (5 injections) (b) or longer (10 injections) (c) treatment with agly-anti-CD3 (5 μ g/day). Differences between groups in (b) and (c) were assessed using the Log Rank survival test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

population at different time-points after transfer showed that anti-CD3 treatment, albeit completely protecting mice from developing diabetes, did not preferentially deplete the originally transferred GFP⁺ cells, as these were present in equal ratios in both anti-CD3-treated and control-treated mice, but rather prevented further activation of subsequent waves of islet-reactive T cells.

Investigation of peripheral blood mononuclear cells in teplizumab recipients has identified differential expression of several genes involved in T-cell activation and effector function.⁴³ In our experiments, GSEA of microarray data of GFP⁺ transferred Th1 cells showed some major differences between treated and untreated cells. Fourteen molecular signatures were strongly associated with cells from untreated mice, compared with nine from anti-CD3-treated mice. T cells from isotype-treated control mice had the transcriptional hallmark of activated cells.

The finding that untreated effector Th1 cells were strongly enriched in transcripts encoding components of the glycolysis pathway suggests that anti-CD3 treatment may in some way suppress metabolic activity or alternatively select or skew T cells to other modes of metabolism such as fatty acid metabolism. This shift in metabolic mode has been well described in Treg cells and memory T cells.^{44,45} A recent report described that signalling through PD-1 leads to down-regulation of glycolysis in T cells,⁴⁶ and in our studies we find that PD-1 is up-regulated in the anti-CD3-treated effector cells. Previous studies have demonstrated that blockade of PD-1/PD-L1 interaction increases the interaction time between T cells and antigen-presenting cells and enhances activation,^{33,47} and expression of PD-L1 is important for regulating anti-islet immune responses.^{28–32} Our results show that anti-CD3 treatment up-regulates PD-1 on effector T cells, and that

anti-CD3 treatment is not effective if the PD-1/PD-L1 pathway is inhibited. In our studies of PD-L1 blockade, both activated islet-specific cells as well as naive cells are potentially affected by the anti-PD-L1, and it remains to be determined if the increase of PD-1 is the most important factor in the anti-CD3-mediated reduction of pathogenicity. It will be interesting to assess further the effects of anti-CD3 on Th1 cellular metabolism, and whether up-regulation of PD-1 precedes the decrease in glycolytic activity. It is also notable that treatment down-regulated pathways involved in co-stimulation of antigen-presenting cells, as well as the PI3K signalling pathway, which is of crucial importance for IFN- γ secretion.^{48,49} The GSEA data support the notion that ex-Th1 cells have been reprogrammed to become less activated or pro-inflammatory. This could be due, at least in part, to the increased ratio of Foxp3⁺ Treg cells present^{11,50} and elevated levels of transforming growth factor- β (TGF- β) in treated recipients,^{13,51,52} as well as to direct effects mediated by the antibody treatment of the effector cell itself. To further investigate this, we investigated the functional properties of the cells from treated animals. We discovered that these ex-Th1 cells were now inhibited from secreting the Th1 hallmark cytokine IFN- γ , which is necessary for islet-specific CD4⁺ T cells to cause diabetes,^{40,42} indicating that treatment had completely changed their behaviour.

The down-regulation of the CD40 pathway suggested by the microarray data indicated that effector cells from treated mice may be deficient in their capacity to licence DC, and so be prevented from perpetuating the anti-islet immune response. In addition, the lack of IFN- γ in anti-CD3-treated ex-Th1 cells would impact on their capacity to licence antigen-presenting cells, as has been demonstrated in studies of CTLA4-immunoglobulin-induced tolerance.²⁷ We found that pre-incubation with agly-anti-CD3 prevented Th1 differentiated BDC2.5 CD4⁺ T cells from activating BMDC in co-culture, both as measured by up-regulation of the co-stimulatory molecule CD80, and secretion of IL-12, which is important to their activating function. We previously found that DC that are unable to deliver full activation signals may, by default, induce tolerance, T-cell unresponsiveness, and Treg cells.⁵³ Foxp3⁺ Treg cells themselves can also inhibit DC function,⁵⁴ an effect that can be broken by additional CD40 ligand stimulation.⁵⁵ Administration of tolerogenic DC potentiates the protective effects of anti-CD3 in allogeneic islet transplantation.⁵⁶ Antigen presentation is influenced by the actions of TGF- β ,^{57,58} the production of which is of crucial importance for the protective effect of anti-CD3 treatment^{13,51} and which can be further amplified through uptake of apoptotic T cells by macrophages.⁵⁹

An interesting aspect of anti-CD3-mediated protection from type 1 diabetes in mice is the importance of timing. Contrary to many other treatment regimens, anti-CD3

treatment is only effective if administered once the mice have become diabetic, and not before.⁶⁰ The effects of anti-CD3 treatment on tolerance to transplanted tissue is also highly dependent on the timing of administration, with treatment coinciding with the priming of alloreactive immune responses (3–7 days after transplant), affording long-term graft survival, which earlier treatment (day 1–3) could not.^{14,50} This may reflect the need for a sufficiently high number of activated islet-reactive T cells to be targeted by anti-CD3 therapy to enable some of the longer-term manifestations necessary for tolerance. The rapid turnover of pathogenic cells seen in our study supports the concept of a crucial treatment window, as the period during which they exert their effect to achieve a perpetuation of the anti-islet immune response is so brief.

In this report we describe how a mouse equivalent of oteixizumab, agly-anti-CD3, prevents pathogenic islet-specific Th1 cells from causing diabetes by inhibiting their metabolism, CD40 signalling and cytokine production, and through up-regulating PD-1 expression. Anti-CD3 does not preferentially deplete these cells, but prevents them from being pathogenic and from contributing to activation of naive cells. Inhibition of PD-1/PD-L1 interaction breaks the protective effect of anti-CD3 treatment in wild-type NOD mice. We propose that inhibition of effector T-cell metabolism and function, and the downstream consequences of this, operates in combination with the favourable ratio of Foxp3⁺ Treg cells and elevated levels of TGF- β to provide the long-term protective effect of the short treatment with aglycosyl-anti-CD3 antibody.

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Disclosures

The authors declare no commercial or financial conflict of interest. Author contributions: MW, HW and AC

designed the studies, MW, AR, JP, CP, MV conducted experiments, and analysed data, DH analysed data, HW, MA and FSW provided reagents, all authors discussed the data and MW wrote the manuscript with assistance from all other authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Properties of agly-anti-CD3.

Figure S2. Injection of agly-anti-CD3 reverses diabetes in non-obese diabetic mice in a dose-dependent manner.

Figure S3. Agly-anti-CD3 treatment increases the percentage of Foxp3⁺ regulatory T (T reg) cells through selectively sparing this population.

Figure S4. Treatment with agly-anti-CD3 does not cause conversion of islet specific T helper type 1 (Th1) effector cells to Foxp3⁺ regulatory T (Treg) cells.

Figure S5. Heatmap presentation of microarray data comparing islet specific T helper type 1 (Th1) cells from agly-anti-CD3 treated and isotype-treated mice.

Figure S6. T helper type 1 (Th1) differentiated cells from agly-anti-CD3-treated recipients do not produce interleukin-10 (IL-10) when restimulated.

Table S1. All the significantly enriched molecular signatures associated with anti-CD3 treated or untreated cells are listed.